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Genetically modified cells and methods for expressing recombinant heparanase and methods of purifying same

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(54) Title: GENETICALLY MODIFIED CELLS AND METHODS FOR EXPRESSING RECOMBINANT HEPARANASE AND METHODS OF PURIFYING SAME

(57) Abstract

Bacterial, yeast and animal cells and methods for overexpressing recombinant heparanase in cellular systems, methods of purifying recombinant heparanase therefrom and modified heparanase species which serve as precursors for generating highly active heparanase by proteolysis.

The claims defining the invention are as follows:

1. A genetically modified cell comprising a polynucleotide sequence encoding a polypeptide cleavable to obtain heparanase catalytic activity, said polynucleotide sequence comprising at least a first nucleotide sequence encoding an N-terminal portion of precursor heparanase, a second, in frame, nucleotide sequence encoding an introduced recognition and cleavage sequence of a protease and a third, in frame, nucleotide sequence encoding a C-terminal portion of heparanase, wherein said recognition and cleavage sequence enables processing of said polypeptide by said protease.

2. The genetically modified cell of claim 1, wherein said first and third nucleotide sequences each correspond to at least a portion of SEQ ID NO: 1.

3. The genetically modified cell of claim 1 or claim 2, wherein said polynucleotide sequence encodes catalytically active heparanase when correctly folded and digested by said protease.

4. The genetically modified cell of any one of claims 1-3, wherein said polynucleotide sequence is stably integrated in the genome of the cell.

5. The genetically modified cell of any one of claims 1-3, wherein said polynucleotide sequence is external to the genome of the cell.

6. The genetically modified cell of any one of claims 1-5, wherein said polynucleotide sequence encodes a signal peptide for protein secretion.

7. The genetically modified cell of any one of claims 1-6, wherein said polynucleotide sequence is selected from the group consisting of double stranded DNA, single stranded DNA and RNA.

8. The genetically modified cell of any one of claims 2-7, wherein said cleaved polypeptide having heparanase catalytic activity is encoded by a portion of SEQ ID NO: 1.

9. The genetically modified cell of any one of claims 1-8, wherein said N- and C-terminal portions of said polypeptide each correspond to at least a portion of SEQ ID NO:2.

10. The genetically modified cell of claim 9, wherein said polypeptide comprises a signal peptide for protein secretion.

11. The genetically modified cell of any one of claims 1-10, wherein said cell is a bacterial cell.

12. The genetically modified cell of claim 11, wherein said cell is E. coli.

13. The genetically modified cell of any one of claims 1-10, wherein said cell is an animal cell..

14. The genetically modified cell of claim 13, wherein said cell is an insect cell.

15. The genetically modified cell of claim 14, wherein said insect cell is selected from the group consisting of High five and Sf21 cells.

16. The genetically modified cell of claim 13, wherein said cell is a mammalian cell.

17. The genetically modified cell of claim 16, wherein said mammalian cell is selected from the group consisting of CHO cells, BHK21 cells, Namalwa cells, Dauidi cells, Raji cells, Human 293 cells, Hela cells, Ehrlich's ascites cells, Sk-Hep1 cells, MDCK-1 cells, MDBK-1 cells, Vero cells, Cos cells, CV-1 cells, NIH3T3 cells, L929 cells and BLG cells.

18. The genetically modified cell of any one of claims 1-10, wherein said cell is a yeast cell.

19. The genetically modified cell of claim 18, wherein said yeast cell is a methylotrophic yeast.

20. The genetically modified cell of claim 18, wherein said yeast cell is selected from the group consisting of Pichia pastoris, Hansenula polymorpha and Saccharomyces cerevisiae.

21. A preparation comprising said polypeptide or said cleaved polypeptide having heparanase catalytic activity, said polypeptide or cleaved polypeptide being obtained from the genetically modified cell of any one of claims 1-20.

22. A polypeptide cleavable to obtain an active heparanase enzyme, said polypeptide being obtained from the genetically modified cell of any one of claims 1-20.

23. A cleaved polypeptide having heparanase catalytic activity obtained from the genetically modified cell of any one of claims 1-20.

24. A precursor heparanase polypeptide when obtained from the genetically modified cell according to any one of claims 1-20.

25. A protease processed heparanase polypeptide when obtained from the genetically modified cell according to any one of claims 1-20.

26. The polypeptide of claim 25, wherein said protease is selected from the group consisting of a cysteine protease, an aspartyl protease, a serine protease and a metalloproteinase.

27. The polypeptide of claim 25 or claim 26, wherein digesting said polypeptide is performed at a pH wherein said protease is active.

28. The polypeptide of claim 27, wherein said protease is most active at said pH.

29. The polypeptide of any one of claims 25-28, wherein said protease does not have a recognition and cleavage sequence in said N- and C- terminal portions of said polypeptide.

30. A recombinant heparanase protein cleavable to obtain heparanase catalytic activity, said protein being encoded by a polynucleotide sequence comprising at least one introduced recognition and cleavage sequence of a protease, wherein said recognition and cleavage sequence enables the processing of said protein by said protease.

31. A method of preparing a polypeptide having heparanase catalytic activity, comprising:

providing a genetically modified cell comprising a polynucleotide sequence encoding a precursor polypeptide having at least one introduced cleavage site cleavable by a protease to obtain a polypeptide having heparanase catalytic activity;

selecting a protease specific for said cleavage site; and

cleaving said precursor polypeptide with said protease to obtain said polypeptide having heparanase catalytic activity.

32. A nucleic acid construct encoding heparanase protein cleavable to obtain heparanase catalytic activity, said construct comprising at least a first nucleic acid sequence encoding an N-terminal portion of precursor heparanase, a second, in frame, nucleic acid sequence encoding an introduced recognition and cleavage sequence of a protease and a third, in frame, nucleic acid sequence encoding a C-terminal portion of heparanase, wherein said recognition and cleavage sequence enables the processing of said protein by said protease.

33. The nucleic acid construct of claim 32, wherein said protease does not have a recognition and cleavage sequence in said N- and C- terminal portions.

34. The nucleic acid construct of claim 32 or claim 33, wherein said nucleic acid sequence encodes a catalytically active heparanase when correctly folded and digested by said protease.

35. A method of activating a heparanase enzyme comprising digesting a non active precursor heparanase enzyme having an introduced protease recognition and cleavage site by a protease.

36. The method of claim 35, wherein said protease is selected from the group consisting of a cysteine protease, an aspartyl protease, a serine protease and a metalloproteinase.

37. The method of claim 35 or claim 36, wherein said digesting is effected at a pH in which said protease is active.

38. The method of claim 37, wherein said protease is most active at said pH.

39. A method of obtaining a substantially purified, active heparanase protein, the method comprising:

expressing a precursor heparanase protein having at least one introduced protease cleavage site in a cell which secretes the precursor heparanase protein into a growth medium to obtain a conditioned growth medium;

treating the precursor heparanase protein with a protease specific for said cleavage site to obtain said active heparanase protein; and

purifying said active heparanase protein.

40. A method of obtaining a substantially purified, active heparanase protein, the method comprising:

expressing a precursor heparanase protein in a cell which secretes the precursor heparanase protein into a growth medium to obtain a conditioned growth medium, the precursor heparanase protein including an N-terminal portion of precursor heparanase,

an introduced recognition and cleavage sequence of a protease and a C-terminal portion of heparanase, wherein the protease is selected for not having a recognition and cleavage sequence in the N- and C-terminal portions of the precursor heparanase protein;

treating the precursor heparanase protein with the protease; and

purifying said active heparanase protein.

41. The method of claim 39 or claim 40, wherein the precursor heparanase protein is obtained from the genetically modified cell according to any one of claims 1-20.

42. The method of any one of claims 39-41, wherein said protease is selected from the group consisting of a cysteine protease, an aspartyl protease, a serine protease and a metalloproteinase.

43. The method of any one of claims 39-42, wherein said treating is effected at a pH in which said protease is active.

44. The method of claim 43, wherein said protease is most active at said pH.

45. A method of obtaining active heparanase protein comprising:

genetically modifying a cell with an expression vector encoding a precursor heparanase protein cleavable to obtain said active heparanase protein, wherein said expression vector includes at least a first nucleic acid sequence encoding an N-terminal portion of precursor heparanase, a second, in frame, nucleic acid sequence encoding an introduced recognition and cleavage sequence of a protease and a third, in frame, nucleic acid sequence encoding a C-terminal portion of heparanase, wherein said recognition and cleavage sequence results in said precursor heparanase protein being processed by said protease.

46. The method of claim 45, wherein said precursor heparanase protein comprises an amino acid sequence as set forth in SEQ ID NO:2 or a biologically active portion thereof.

47. The method of claim 45 or claim 46, wherein said precursor heparanase protein is encoded by a nucleotide sequence according to SEQ ID NO: 1 or a portion thereof.

48. The method of any one of claims 45-47, wherein said expression vector encodes a catalytically active heparanase when correctly folded and digested by said protease.

49. The method of any one of claims 45-48, wherein said cell is a bacterial cell.

50. The method of claim 49, wherein said cell is E. coli.

51. The method of any one of claims 45-48, wherein said cell is an animal cell.

52. The method of claim 51, wherein said cell is an insect cell.

53. The method of claim 51, wherein said cell is a mammalian cell.

54. The method of claim 53, wherein said mammalian cell is selected from the group consisting of CHO cells, BHK21 cells, Namalwa cells, Dauidi cells, Raji cells, Human 293 cells, Hela cells, Ehrlich's ascites cells, Sk-Hep1 cells, MDCK-1 cells, MDBK-1 cells, Vero cells, Cos cells, CV-1 cells, NIH3T3 cells, L929 cells and BLG cells.

55. The method of any one of claims 45-48, wherein said cell is a yeast cell.

56. The method of claim 55, wherein said yeast cell is a methylotrophic yeast.

57. The method of claim 55, wherein said yeast cell is selected from the group consisting of Pichia pastoris, Hansenula polymorpha and Saccharomyces cerevisiae.

58. A method of obtaining active heparanase protein from precursor heparanase protein having an introduced protease cleavage site which is cleavable to produce said active heparanase protein, said method comprising:

genetically modifying a cell with an expression vector encoding said precursor heparanase protein;

growing said cell in growth medium;

subjecting said cell to a substance which induces secretion of said precursor heparanase protein into the growth medium; and

treating the precursor heparanase protein with a protease specific for said cleavage site to obtain said active heparanase protein.

59. The method of claim 58, wherein said substance is selected from the group consisting of thrombin, calcium ionophores, immune complexes, antigens and mitogens.

60. The method of claim 59, wherein said calcium ionophore is calcimycin.

61. The method of claim 59, wherein said substance is phorbol 12-myristate 13-acetate.

62. The method of any one of claims 45-61, wherein said cell is grown in a large biotechnological scale of at least half a liter growth medium.

63. The method of any one of claims 45-62, further comprising purifying said active heparanase protein.

64. The method of claim 63, wherein said purification is effected in part by an ion-exchange column.

65. The method of claim 64, wherein said ion-exchange column is a Source-S column.

66. The method of any one of claims 63-65, wherein said purification is from a preparation containing said cell.

67. The method of any one of claims 63-65, wherein said purification is from a growth medium in which said cell is grown.

68. The method of any one of claims 63-65, wherein said purification is from an extract of a culture derived of said cell.

69. A method of activating a mammalian heparanase protein, the method comprising digesting the mammalian heparanase protein with a protease capable of cleaving said mammalian heparanase protein at a region containing its most hydrophilic sites present within the first 170 N terminal amino acids of said mammalian heparanase protein, as determined using the Kyte-Doolittle method for calculating hydrophilicity, using the Wisconsin University GCG DNA analysis software, so as to release a catalytically active portion of said mammalian heparanase.

70. A method of activating a modified mammalian heparanase protein, wherein the modified mammalian heparanase protein comprises at least one introduced protease cleavage recognition sequence at a region containing the most hydrophilic sites present within the first 170 N terminal amino acids of a natural mammalian heparanase protein as determined using the Kyte-Doolittle method for calculating hydrophilicity, using the Wisconsin University GCG DNA analysis software, the method comprising digesting the modified mammalian heparanase protein with a matching protease being capable of cleaving said introduced protease cleavage recognition sequence.

71. The method of claim 70, wherein said protease is selected from the group consisting of a cysteine protease, an aspartyl protease, a serine protease and a metalloproteinase.

72. The method of claim 70 or claim 71, wherein digesting the modified mammalian heparanase protein by said matching protease is effected at a pH in which said protease is active.

73. The method of claim 72, wherein digesting the modified mammalian heparanase protein by said matching protease is effected at a pH in which said protease has an optimal protease activity.

74. A genetically modified cell as defined in claim 1 and substantially as hereinbefore described with reference to Example 6.

75. A recombinant heparanase protein as defined in claim 30 and substantially as hereinbefore described with reference to Example 6.

76. A nucleic acid construct as defined in claim 32 and substantially as hereinbefore described with reference to Example 6.

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By their Patent Attorneys
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